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Lipid-Peroxidation (methodischer Artikel)

3. After centrifugation at 3000 rpm for 10 min, 0.5 ml of the supernatant is transferred to another centrifuge tube. In the case of the serum, 20 μl of the specimen is taken.

4. To this solution, 4.0 ml of N/12 H_2SO_4 is added and the mixture is shaken gently.

5. Then, 0.5 ml of 10% phosphotungstic acid is added and mixed. After standing at room temperature for 5 min, the mixture is centrifuged at 3000 rpm for 10 min.

6. The supernatant is discarded, and the sediment is mixed with 2.0 ml of N/12 H_2SO_4 and 0.3 ml of 10% phosphotungstic acid. The mixture is centrifuged at 3000 rpm for 10 min.

7. The sediment is suspended in 4.0 ml of distilled water, and 1.0 ml of TBA reagent is added. The reaction mixture is heated for 60 min at 95° in an oil bath.

8. After cooling with tap water, 5.0 ml of *n*-butanol is added and the mixture is shaken vigorously.

9. After centrifugation at 3000 rpm for 15 min, the *n*-butanol layer is taken for fluorometric measurement at 553 nm with 515 nm excitation.

10. Taking the fluorescence intensity of the standard solution, which is obtained by reacting 0.5 nmol of tetramethoxypropane with TBA by steps 7–9, as F and that of the sample as f, the lipid peroxide level (Lp) can be expressed in terms of malondialdehyde:

$$\text{Plasma } Lp = 0.5 \times \frac{f}{F} \times \frac{1.05}{0.05} \times \frac{1.0}{0.5} = \frac{f}{F} \times 21 \qquad \text{(nmol/ml of blood)}$$

$$\text{Serum } Lp = 0.5 \times \frac{f}{F} \times \frac{1.0}{0.02} = \frac{f}{F} \times 25 \qquad \text{(nmol/ml of serum)}$$

[40] Spectrophotometric Detection of Lipid Conjugated Dienes

By RICHARD O. RECKNAGEL and ERIC A. GLENDE, JR.

Principles of the Method

Like many other substances, naturally occurring lipids exhibit simple end absorption in ultraviolet light as the wavelength is lowered toward 200 nm (Figs. 1 and 2). The spectra of a variety of organic molecules contain-

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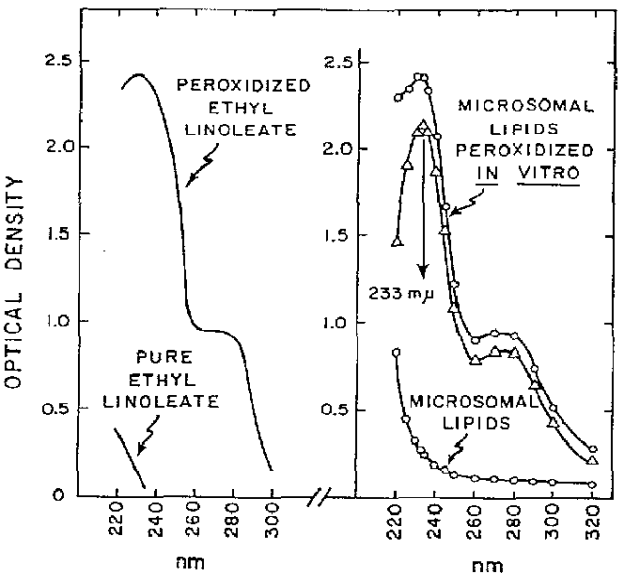

FIG. 1. Conjugated diene absorption of peroxidized ethyl linoleate and rat liver microsomal lipids peroxidized *in vitro*. The lower trace of the right panel is the end absorption of nonperoxidized microsomal lipids. The upper trace of the right panel is the absorption spectrum of peroxidized microsomal lipids. The middle trace is the difference spectrum. Left panel: from J. L. Bolland and H. P. Koch, *J. Chem. Soc.* 445 (1945). From R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* **15**, 132 (1966); reproduced with permission.

ing conjugated dienes, however, are characterized by intense absorption, the so-called K band, which may range, with respect to peak absorption, from 215 to 250 nm, depending on nearby substituent groups.[1,2] The spectra of peroxidized lipids are characterized by an intense K band near 233 nm, with a lesser secondary absorption maximum due to ketone dienes, in the region 260–280 nm. The appearance of conjugated dienes in peroxidized unsaturated fatty acids is due to resonance following free radical attack on hydrogens of methylene groups separating double bonds in these compounds.[3] Since the absorption at 233 nm due to conjugated dienes is superimposed on the end absorption of the nonperoxidized lipid,

[1] R. B. Woodward, *J. Am. Chem. Soc.* **63**, 1123 (1941).
[2] R. B. Woodward, *J. Am. Chem. Soc.* **64**, 72 (1942).
[3] R. O. Recknagel, E. A. Glende, Jr., R. L. Waller, and K. Lowrey, *in* "Toxicology of the Liver" (G. Plaa and W. R. Hewitt, eds.), p. 213. Raven, New York, 1982.

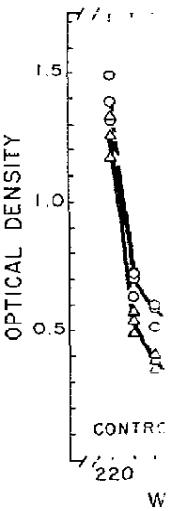

FIG. 2. Conjugated diene absorpti
administration of CCl_4. The lower t
lipids from normal untreated rats. Fr
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one principle of the method i
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absorption of a correspondin
control (Figs. 1 and 2).

Ultraviolet spectrophome
used for many years in the
lipids.[4] The method appears t
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been widely used since. Inc
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of membrane lipids has oc
method recognizes that for w
peroxidation, the fraction of
not only be low, but the proc
particular subcellular structu

[4] R. T. Holman, *in* "Progress in the
W. O. Lundberg, and T. Malkin,
[5] R. O. Recknagel and A. K. Ghos

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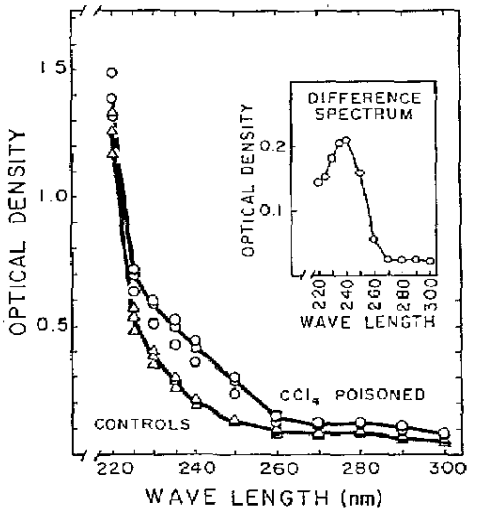

FIG. 2. Conjugated diene absorption in rat liver microsomal lipids 5 min after intragastric administration of CCl_4. The lower trace is end absorption of nonperoxidized microsomal lipids from normal untreated rats. From K. S. Rao and R. O. Recknagel, *Exp. Mol. Pathol.* 9, 271 (1968); reproduced with permission.

one principle of the method involves determination of the difference between the 233 nm absorption of the peroxidized lipid and the 233 nm absorption of a corresponding sample of nonperoxidized lipid serving as control (Figs. 1 and 2).

Ultraviolet spectrophometric detection of conjugated dienes has been used for many years in the food industry for detection of autoxidized lipids.[4] The method appears to have been applied for the first time to the problem of liver cell lipid peroxidation of toxigenic origin in 1966[5] and has been widely used since. Increasingly, for a variety of pathological processes, the question has been raised whether peroxidative decomposition of membrane lipids has occurred *in vivo*. A second principle of the method recognizes that for whole-animal studies involving possible lipid peroxidation, the fraction of endogenous lipids actually peroxidized may not only be low, but the process of lipid peroxidation may be confined to a particular subcellular structure, e.g., the endoplasmic reticulum (ER) of

[4] R. T. Holman, *in* "Progress in the Chemistry of Fats and Other Lipids" (R. T. Holman, W. O. Lundberg, and T. Malkin, eds.), Vol. 2, p. 51. Pergamon, Oxford, 1954.

[5] R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* 15, 132 (1966).

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rat liver cells after CCl_4 intoxication. The end absorption of nonperoxidized lipids from cellular components unaffected by the pathological process under investigation may mask the absorption at 233 nm. It is for this reason that the starting point for detection of lipid conjugated dienes is homogenization of the tissue followed by differential centrifugation to isolate the major subcellular fractions. All aqueous phase procedures are conducted in the presence of ethylenediaminetetraacetate (EDTA) or other Fe^{2+}-chelating agents to avoid possible lipid peroxidation during homogenization and centrifugation procedures. Total lipids of a particular isolated subcellular fraction can be extracted conveniently with chloroform–methanol 2 : 1, according to Folch *et al.*[6] with modifications introduced by Bligh and Dyer.[7] Extracted lipids in chloroform are eventually dried down by removing the chloroform under a stream of oxygen-free nitrogen, or in suitable evaporation devices. Absolutely oxygen-free nitrogen can be obtained by passing commercial "oxygen-free" nitrogen over hot reduced copper mesh. The dried-down and chloroform-free lipid is dissolved in spectrophotometric grade cyclohexane for spectrophotometric analysis. The final concentration of lipid in cyclohexane can be determined according to Chiang *et al.*[8] All absorbance measurements are then corrected to a uniform base of 1 mg lipid per ml of cyclohexane.

According to this method, estimation of lipid peroxidation *in vivo* depends on determination of the difference spectrum at 233 nm for lipids from control animals and lipids from treated animals. Since the difference spectrum depends on two measured quantities, error in the difference spectrum will be greater than for each of the measured quantities alone. Because of this tendency for greater error in the difference spectrum, we routinely obtain a mean difference spectrum, with at least three animals in the control group and three animals in the experimental group. Finally, since the spectrophotometric readings are adjusted to a final concentration of 0.1 g% at the time of assay, the mean difference spectrum is multiplied by 10 to obtain the mean difference spectrum at a concentration of 1%. This yields the so-called mean delta $E^{1\%}_{1\,cm}$ value.[9]

The shift of the double bond which occurs as a result of radical attack on polyenoic fatty acids can take place under special circumstances in the absence of molecular O_2, and hence in the absence of peroxidation reactions per se. For example, conjugated dienes appear in the lipids of rat liver microsomes that have been incubated anaerobically in the presence

[6] J. Folch, M. Lees, and G. H. Sloane-Stanley, *J. Biol. Chem.* **226**, 497 (1957).
[7] E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
[8] S. P. Chiang, C. F. Gessert, and O. H. Lowry, in Research Report 56-113, Air University School of Aviation Medicine, p. 1, USAF, Texas, 1957.
[9] R. O. Recknagel and A. K. Ghoshal, *Exp. Mol. Pathol.* **5**, 413 (1966).

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double bond. This initial p
However, the rapid reacti
that peroxidation reaction
functions, will occur in ae
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present.

Reagents

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Methanol
Chloroform–methano
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Cyclohexane, spectro
O_2-free nitrogen. Com
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Details of Procedure

Although the following
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somes is carried out in co
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red quantitatively with 6
e.g., a graduated, 40-ml h
No. 8144). The volume i

[10] C. L. Wood, A. J. Gandolfi,
[11] G. R. McMillan and J. G. C

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of NADPH and halothane[10] or NADPH and CCl_4.[10] Evidently, generation of radical products from halocarbon substrates can lead to the shift in the double bond. This initial part of the overall process does not require O_2. However, the rapid reaction of lipid organic radicals with O_2[11] will ensure that peroxidation reactions, in the strict sense of formation of peroxy functions, will occur in aerobic systems where polyenoic fatty acids are attacked by radicals. It is for this reason that the shift of the double bond, which does not in and of itself involve peroxide formation, is nevertheless a clear sign that lipid peroxidation has occurred, if molecular O_2 is present.

Reagents

Chloroform
Methanol
Chloroform–methanol, 2 : 1, v/v. We use commercially available reagent grades of these solvents without further purification
Cyclohexane, spectrophotometric grade
O_2-free nitrogen. Commercial "oxygen-free" nitrogen is passed over hot, reduced copper mesh to remove the last traces of oxygen. The hot copper mesh is reduced with hydrogen gas. Alternatively, several brands of oxygen traps, originally designed for purifying carrier gases in gas–liquid chromatography, are available from chromatography supply houses. Some of these do not incorporate an indicator of exhaustion.

Details of Procedure

Although the following procedure is written for detection of lipid conjugated dienes in the microsome fraction obtained from rat liver, it is equally applicable to any isolated subcellular component, tissue, etc.

1. The microsome fraction is obtained by centrifugation of liver homogenates prepared in media (isotonic sucrose, saline-phosphate buffer, etc.) containing at least 0.001 *M* EDTA. Final sedimentation of the microsomes is carried out in centrifuge tubes of a type which resists methanol and thereby facilitates the next step. The final supernatant fraction and any fat clinging to the walls of the centrifuge tube are discarded. The sedimented microsomes (usually from 2 to 3 g of whole liver) are transferred quantitatively with 6–7 ml methanol to a suitable graduated container, e.g., a graduated, 40-ml heavy-walled, stoppered centrifuge tube (Corning No. 8144). The volume is adjusted to 7 ml with methanol.

[10] C. L. Wood, A. J. Gandolfi, and R. A. Van Dyke, *Drug Metab. Dispos.* **4**, 305 (1976).
[11] G. R. McMillan and J. G. Calvert, *Oxid. Combust. Rev.* **1**, 84 (1965).

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2. Add 14 ml chloroform, stopper, and mix thoroughly. Allow the mixture to stand at room temperature for about 10 min with occasional mixing.

3. Centrifuge the mixture at approximately 260 *g* for 10 min at room temperature to sediment the insoluble material. Decant the supernatant fraction into another 40-ml graduated, stoppered centrifuge tube. Adjust the final volume of the lipid extract to 30 ml by the addition of $CHCl_3 : CH_3OH$ (2 : 1, v : v). Alternatively, insoluble material in the original $CHCl_3 : CH_3OH$ extract can be removed by filtration, e.g., through a medium-porosity sintered glass funnel.

4. Add 10 ml H_2O. Mix gently by inversion only. Too vigorous mixing at this point results in emulsions which sometimes are difficult to break.

5. Centrifuge 10 min at approximately 260 *g*.

6. By means of aspiration using a water vacuum or house vacuum remove the upper CH_3OH–water phase and any fluffy material at the interface between the two phases. Note: At this state some workers place the tubes in an ice bath for 5 min, recentrifuge, and aspirate off any remaining CH_3OH–water droplets.

7. Transfer a 2-ml aliquot of the chloroform phase into a clean tube, place in a water bath at 40–50°, and remove the chloroform under a stream of oxygen-free nitrogen. All traces of chloroform must be removed. At this step, some workers dissolve the dried-down lipid in several milliliters of fresh chloroform, transfer to a clean tube, and dry down again.

8. Dissolve the extracted, chloroform-free lipid in 3 ml of cyclohexane and record optical density (1 cm light path) from 300 to 220 nm, against a cyclohexane blank.

9. After the optical density measurements remove 0.25-ml aliquots from each sample for analysis of total lipid content, according to Chiang *et al.*[8]

10. Correct all optical density measurements to a uniform base of 1 mg lipid per ml of cyclohexane.

General note: Once the microsome fraction is obtained, we usually carry out the procedure through step 8 without overnight delay. If necessary, the lipid in cyclohexane can be kept frozen for subsequent analysis.

Detection of Lipid Conjugated Dienes with [^{14}C]Tetracyanoethylene

Quantitative determination of conjugated diene unsaturation can be carried out with [^{14}C]tetracyanoethylene in a Diels–Adler condensation.[12]

[12] R. L. Waller and R. O. Recknagel, *Lipids* 12, 914 (1977).

This method was used for
triglycerides, phospholipids.
^{14}C found in the Diels–Adl
conjugated diene content. A
more difficult and time consu
of conjugated dienes in su
cyanoethylene is not comme
sensitive than the spectropl
tained with this method dem
sorption in the 230 to 235
lipids after CCl_4 or $BrCCl_3$ a
but is actually due to appea
side chains of the tissue lip
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simple method of UV spectr
herein described, is a reliab
merization in the fatty acid
in vivo.

[41] Fluorescent Dam

By CORA J. I

Free radical chain reacti
tion,[1] lead to the formation
many secondary products. A
hyde, malonaldehyde. Malc
groups on biological compo
phospholipids, nucleic acids,
iminopropenes, which are flu
morphic system responsible
When ribonuclease reacts w
with malonaldehyde, it beco
ucts are formed.[3] The spectr
are nearly identical with the

[1] E. Sawiki, T. W. Stanley, and H.
[2] K. S. Chio and A. L. Tappel. *Bio*
[3] K. S. Chio and A. L. Tappel. *Bio*

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This method was used for quantitative analysis of conjugated dienes in triglycerides, phospholipids, and peroxidized tissue lipids. The amount of ^{14}C found in the Diels–Adler adducts was shown to be a measure of conjugated diene content. A disadvantage of this method is that it is much more difficult and time consuming than spectrophotometric determination of conjugated dienes in suitably extracted lipids. Further, [^{14}C]tetracyanoethylene is not commercially available, and the method is no more sensitive than the spectrophotometric method. However, the data obtained with this method demonstrated rigorously that the increase in absorption in the 230 to 235 nm region observed in rat liver microsomal lipids after CCl_4 or $BrCCl_3$ administration is not some nonspecific artifact, but is actually due to appearance of conjugated dienes in the fatty acid side chains of the tissue lipids. The work with [^{14}C]tetracyanoethylene permitted drawing of the important conclusion that the fast and relatively simple method of UV spectrophotometric scanning of extracted lipids, as herein described, is a reliable method for detection of double-bond isomerization in the fatty acid side chains of membrane lipids peroxidized *in vivo*.

[41] Fluorescent Damage Products of Lipid Peroxidation

By CORA J. DILLARD and AL L. TAPPEL

Free radical chain reactions, which take place during lipid peroxidation,[1] lead to the formation of lipid hydroperoxides that decompose to many secondary products. Among the products is a three-carbon dialdehyde, malonaldehyde. Malonaldehyde readily reacts with free amino groups on biological compounds such as amino acids, proteins, amino phospholipids, nucleic acids, etc., to yield *N,N'*-disubstituted 1-amino-3-iminopropenes, which are fluorescent conjugated Schiff bases.[2] The chromorphic system responsible for the fluorescence is N—C=C—C=N. When ribonuclease reacts with peroxidizing polyunsaturated lipid and with malonaldehyde, it becomes inactivated and yellow fluorescent products are formed.[3] The spectral properties of these chromophoric systems are nearly identical with those in chloroform–methanol extracts of age

[1] E. Sawiki, T. W. Stanley, and H. Johnson, *Anal. Biochem.* 35, 199 (1963).
[2] K. S. Chio and A. L. Tappel, *Biochemistry* 8, 2821 (1969).
[3] K. S. Chio and A. L. Tappel, *Biochemistry* 8, 2827 (1969).

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